The Pigments of Cottonseed[1]

CHARLOTTE H. BOATNER
Southern Regional Research Laboratory, New Orleans, Louisiana
Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration,
U. S. Department of Agriculture

Introduction

The presence of dark unstable pigments in cottonseed has always constituted a problem in the utilization of this material as a source of oil and protein. The first scientific study, by Kuhlmann, of the pigments of cottonseed dates as far back as 1861 (1). In spite of this lapse of time only one pigment, namely gossypol, has ever been isolated and its chemical properties determined. This is the more surprising in view of the fact that gossypol is one of the lightest colored of the evidently large variety of pigments which occur naturally in cottonseed.

Gossypol, a yellow pigment of cottonseed, has been investigated by a large number of workers since its first isolation and characterization by Marchlewski in 1899 (2). This early work showed that gossypol is a polyphenolic compound having two carbonyl groups and possesses the interesting property of forming compounds with both acids and bases. Interest in this complex, unstable compound was revived by the discovery in 1915 by Withers and Carruth (3) that gossypol alone could produce the symptoms associated with the so-called cottonseed injury produced in livestock by the feeding of cottonseed in large quantities. Following this discovery Carruth and co-workers instituted an investigation of the chemical properties of gossypol. Carruth later found (4) that the reduced toxicity of cottonseed meal brought about by heat treatment could be correlated with a reduced content of extractable gossypol. Clark (5) published a series of articles on gossypol derivatives in which he showed among other things that gossypol can be extracted with aniline from "heat detoxified" cottonseed meal. By analogy to the reaction with aniline Clark proposed (6) that the "bound" gossypol was gossypol that had combined with the protein of the cottonseed, and thus had been rendered non-toxic and unextractable with ordinary solvents. Karrer and Tobler (7) included a study of gossypol in their series of investigations of plant pigments and Schmid and Margulies (8) investigated the basic structure of gossypol and showed it to be a napthalene derivative. Recently, Adams and a number of co-workers published a series of articles on the structure of gossypol (9).

Thus far, it has not been possible to synthesize gossypol or related products other than the simplest degradation products, hence its complete structure has not been established. It has, however, been shown conclusively (10) that gossypol is a hexahydroxybinaphthalene compound having as substituents two carbonyl groups and two isopropyl groups. In order to explain the great variety of its reactions, Adams and co-workers (11) proposed a structure for gossypol which exists in three tautomeric forms as shown in Figure 1.

The results reported here were obtained in the course of an investigation having as its immediate objective the establishment of a chemical basis for the color changes occurring in cottonseed during storage. With an accurate knowledge of the individual pigments responsible for the color of cottonseed, it may be expected that not only will the changes in the whole system be better understood, but also it should be possible to better control the system and thereby avoid undesirable color changes.

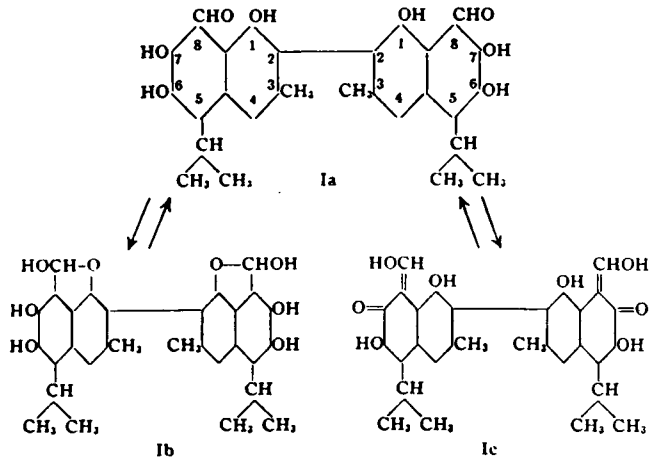

FIG. 1. Structure of gossypol according to Adams.

It has been possible in the course of this investigation to demonstrate the existence of three pigments in cottonseed in addition to gossypol: a red pigment, a yellow oil-soluble pigment, and a purple pigment. The red pigment which accompanies gossypol in most preparations and which develops during the storage of cottonseed has been shown to be an oxidation product of gossypol. Evidence has been found for the existence of an additional yellow pigment which is soluble in oil and not extractable by alkali. The latter pigment is responsible for some, if not all, of the color of alkali-refined cottonseed oil. A purple, unstable pigment, for which the name "gossypurpurin" is proposed, has been isolated and some of its properties have been determined.

Experimental

Preparation and properties of gossypol: Pure gossypol was prepared by an improved procedure which introduced certain modifications in the method of Carruth (12) as modified by Campbell, Morris and Adams (13). Defatted cottonseed meal was extracted in a Soxhlet apparatus with peroxide-free ether. The ether was removed under reduced pressure and the residue was treated with glacial acetic acid. After treatment for twenty-four hours in the cold, the red precipitate which had formed was removed by filtration. Two recrystallizations of the gossypol-acetic acid complex, and two recrystallizations of the regenerated gossypol according to the technique of Carruth and Campbell, Morris and Adams, failed to remove all traces of the red pigment which had precipitated with the gossypol-acetic acid complex from the original mix-

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ture in glacial acetic acid. The technique of Campbell, Morris and Adams, and Carruth consisted of adding acetic acid to the cottonseed extract from which the ether had been only partially removed. The mixture was then allowed to stand in an open container so that most of the remaining ether evaporated. When this procedure was used, the gossypol-acetic acid complex precipitated with even more of the red pigment.

A toluene solution of the repeatedly recrystallized gossypol preparation was passed through a column of 20- to 40-mesh activated tricalcium phosphate.[2] Only in a few instances were well-defined purple, red, and yellow zones observable, but elution with diethyl ether or with toluene removed the pure yellow gossypol and left the mixed red and purple pigments on the column.

The absorption spectrum[3] in the range of visible and near ultraviolet wave lengths of a freshly prepared ethyl alcohol solution of the gossypol purified by the calcium phosphate treatment showed an absorption maximum at approximately 365 m ($m\mu$) : $E\ \frac{1\%}{1\ cm.} = 390$; $E\ \frac{molar}{1\ cm.} = 16.1 \times 10_3$. The absorption maximum occurred at the same wave length for ether and chloroform solutions of this gossypol preparation. With a Coleman double monochromator spectrophotometer it was not possible to find the exact location of the absorption maximum, but with an instrument which isolates a narrower band (14), Dr. F. P. Zscheile, Jr. found that the maximum occurs at 3660 ± 10 Å in ether (15).

As shown in Figure 2, comparison of the absorption spectrum of the purified gossypol freshly dissolved in 95-percent ethyl alcohol with those reported by Adams and Kirkpatrick (16), and Grünbaumowna and Marchlewski (17) shows that their preparations contained more of the red pigment as an impurity than is the case with our preparation. The curves for Adams' and Marchlewski's absorption spectra were replotted from their published data. Marchlewski's absorption data were obtained with an ethyl alcohol solution of the gossypol-acetic acid complex. Measurements in this laboratory indicate that the acetic acid binding does not affect the spectrum of gossypol.

Red oxidation product of gossypol: During the purification of gossypol by precipitation of the gossypol-acetic acid complex from glacial acetic acid it was observed that the supernatant acetic acid was deep red brown in color. By washing the gossypol-acetic acid complex with acetic acid more of the red could be removed from the precipitate. The absorption spectra of the red acetic acid supernatant and wash liquid differ markedly from those of either the original extract or the purified gossypol-acetic acid complex. The absorption spectrum of the original extract exhibits three well-defined absorption maxima at 365, 525 and 560 m ($m\mu$) and absorption is high in the region of 450 to 500 m ($m\mu$). The pure gossypol-

[2] Obtained from Victor Chemical Works, Chicago, Illinois.

[3] All absorption data reported here are expressed in terms of the extinction coefficient, E, since there is a direct linear relation between the extinction coefficient and the concentration of a solution for any given wavelength. This relation is shown by the equation: $E = \frac{\log Io/I}{cl}$ where Io is the intensity of light transmitted by pure solvent, I the intensity of light transmitted by the solution, c the concentration of the solution and l the length in centimeters of the path of light through the liquid. $E\ \frac{molar}{1\ cm.}$ and $E\ \frac{1\%}{1\ cm.}$ are the extinction coefficients computed for molar and 1 percent solutions, respectively.

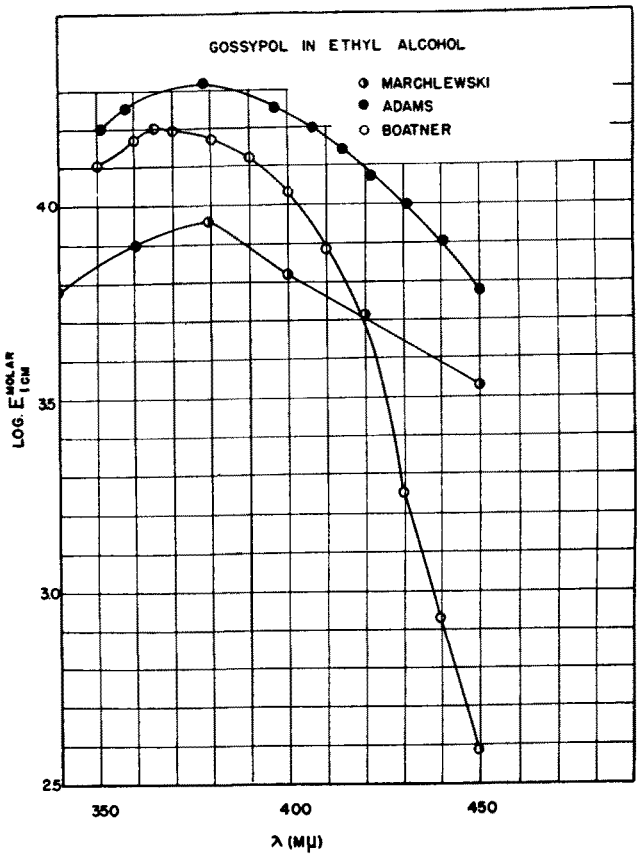

FIG. 2. Absorption spectra of gossypol in ethyl alcohol.

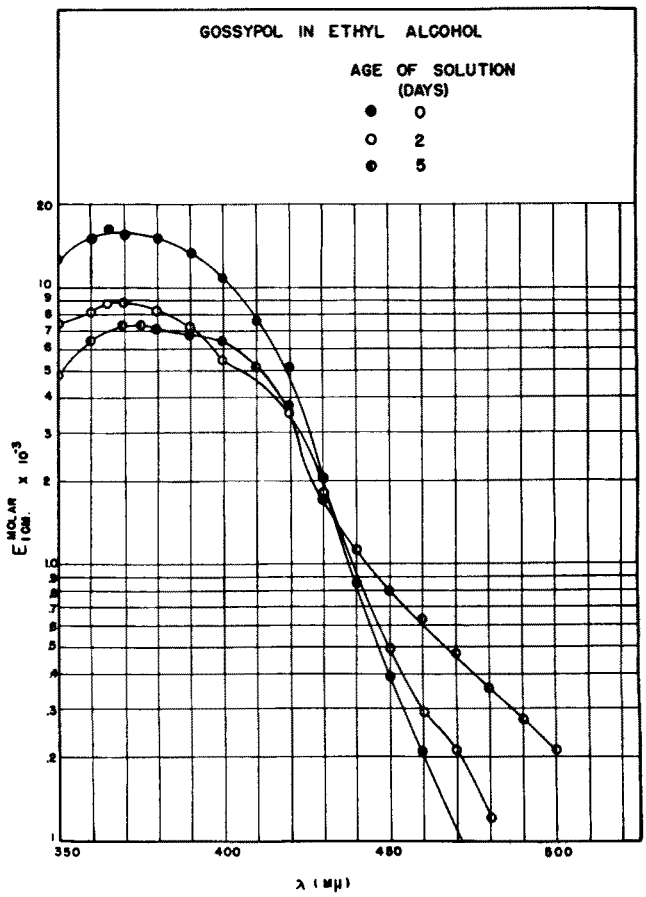

FIG. 3. Change in absorption spectrum of gossypol on standing in ethyl alcohol.

acetic acid complex has one sharp absorption maximum at 365 m ($m\mu$) and practically no absorption in the region of 450 to 500 m ($m\mu$), whereas the absorption spectrum of the material soluble in acetic acid shows a continuous logarithmic increase in the direction of the shorter wave lengths. The fact that repeated washing of the gossypol-acetic acid complex with acetic acid effects a gradual decrease in the absorption in the region of 450 to 500 m ($m\mu$) indicates that the absorption maximum of the pure red pigment is located in this region. An absorption maximum in this wave length region is characteristic of 1,2-naphthoquinones (18).

Evidence concerning the origin of the red pigment was obtained by observing the changes in the absorption spectrum which occurred when solutions of yellow gossypol in ethyl alcohol or in chloroform were allowed to stand in contact with air for some time. In both solvents the absorption in the longer wave length region increased markedly as is evident in Figures 3 and 4.

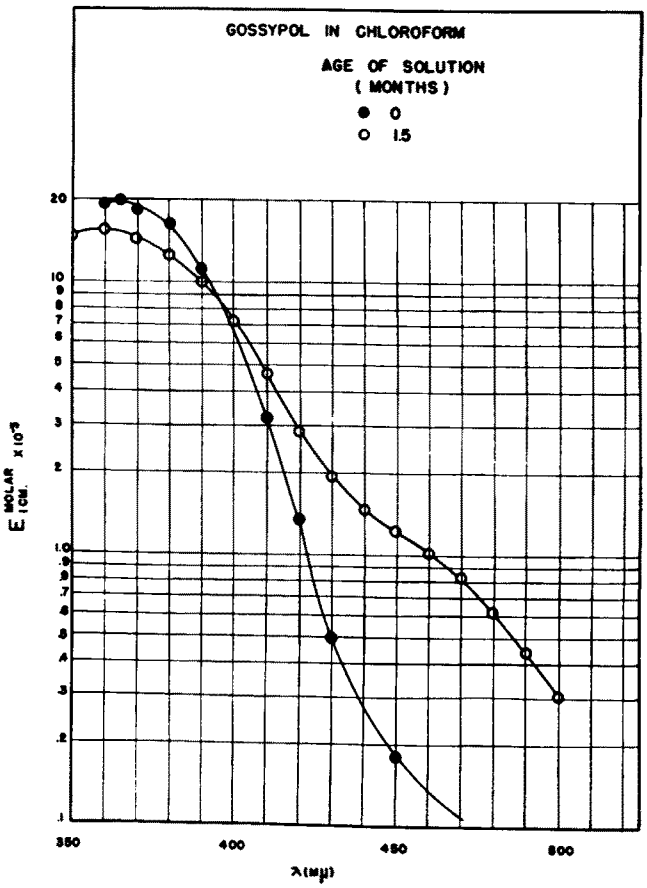

FIG. 4. Change in absorption spectrum of gossypol on standing in chloroform.

The red pigment, some of which precipitates from acetic acid with the gossypol-acetic acid complex, but most of which remains dissolved in acetic acid, is evidently an oxidation product of gossypol. The fact that the color can be reduced by treatment with sodium dithionite indicates that it is probably a quinone. The red color observed by Campbell, Morris and Adams (13) when solutions of either gossypol or so-called "red gossypol" (19) were treated with dry hydrogen chloride was evidently due to the reaction of the red pigment, as this reaction was not observed with the purified gossypol reported here. The fact that it can be extracted along with gossypol by aqueous sodium bicarbonate demonstrates that it is probably acidic in nature. It is probable, therefore, that the red pigment is a quinoid oxidation product of gossypol in which the acidic groups have not been affected. Since this pigment is probably responsible for many of the observed color changes which occur during cottonseed storage (20), efforts are being made to obtain it in pure form in order to determine its properties.

Colorimetric test for gossypol: All of the published methods for the quantitative determination of gossypol in cottonseed and cottonseed oil are gravimetric and, because of the peculiar properties of gossypol, they are very tedious and time consuming. One of the most rapid methods (21) requires seventy-two hours for the complete precipitation of dianilino-dipyridine gossypol on the basis of which the gossypol content is calculated. The reaction of gossypol with concentrated sulfuric acid to form a red solution is the qualitative test usually used for the detection of gossypol, but in many cases deeply colored decomposition products of the other components of cottonseed obscure the gossypol color test. In connection with the determination of the relationship between gossypol and the increase in the color of cottonseed during storage, a rapid method for the estimation of gossypol was required.

It was observed that gossypol formed a brilliant red color when treated with a chloroform solution of antimony trichloride, and an examination of the absorption spectrum of this reaction product showed the presence of a broad and reproducible absorption maximum at 510 to 520 m ($m\mu$). The color reaches its maximum development in less than five minutes and is stable for at least four days. For fixed ratios of antimony trichloride and gossypol solution the extinction is directly proportional to the concentration of the gossypol for concentrations of gossypol ranging from 0.0005 to 0.0025 percent. At higher or lower concentrations the absorption is too high or too low to be read accurately with the spectrophotometer.

That the absorption maximum at 510-520 m ($m\mu$) is probably specific for gossypol, and is not obtained with any of the other pigments extracted by ether or chloroform from cottonseed meal, is evident from the absorption curves in Figure 5. Consequently, the reaction can serve as a qualitative and semi-quantitative test for gossypol. However, since the curves are not perfectly parallel in the region of 420 to 600 m ($m\mu$), methods for removing or determining the interfering reactants are being investigated in order that the extinction coefficient at 510 m ($m\mu$) of the antimony trichloride reaction can be used as a strictly quantitative measure of extractable gossypol.

Yellow oil-soluble pigment of cottonseed: During the development of the antimony trichloride test for gossypol in cottonseed meal, it was observed that contrary to the results obtained with ether, chloroform, and ethylene chloride extracts of cottonseed meal, Skellysolve F extracts of cottonseed meal gave a reaction product with an entirely different absorption curve than that observed with pure gossypol and antimony trichloride (Figure 6). It appeared that an additional pigment extracted by Skellysolve F also gave a characteristic color reaction with antimony trichloride which obscured the gossypol color reaction.

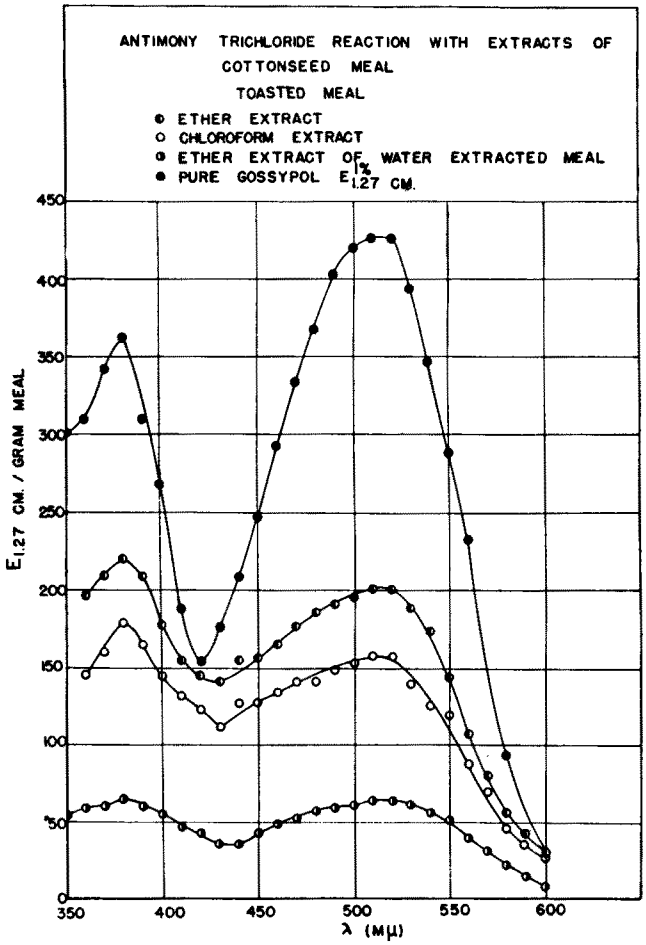

FIG. 5. Absorption spectra of antimony trichloride reaction products with gossypol and with extracts of cottonseed meal.

If the additional pigment were extracted by both ether and Skellysolve F then the sum of the spectrum of a Skellysolve F extract and the spectrum of an ether extract of the Skellysolve F extracted meal should be identical with the spectrum obtained from an ether extract of the whole undefatted meal. That this was not the case is shown in Figure 6. The curves shown in this figure indicate that Skellysolve F extracts a pigment which is not extracted to any appreciable extent by ether and that this pigment is responsible for the anomalous reaction of antimony trichloride with Skellysolve F extracts of cottonseed meal.

The nature of the absorption spectrum of the reaction product of this pigment with antimony trichloride could be estimated by subtracting from the curve obtained for the reaction of the Skellysolve F extract and antimony trichloride the curve calculated for the gossypol reaction product. The calculation of the gossypol curve was based on the assumption that all of the absorption at 510 m (mμ) of the Skellysolve F extract was due to the gossypol-antimony trichloride reaction product. The spectrum obtained by this means is shown in Figure 7. In the same figure are shown the spectra of the antimony trichloride reaction products with oils from another batch of cottonseed after the oils had been refined by alkali extraction. Alkali extraction removes gossypol as well as other acidic components of cottonseed oil. It is evident from these spectra that there is an additional oil-soluble, non-acidic, yellow pigment which reacts with

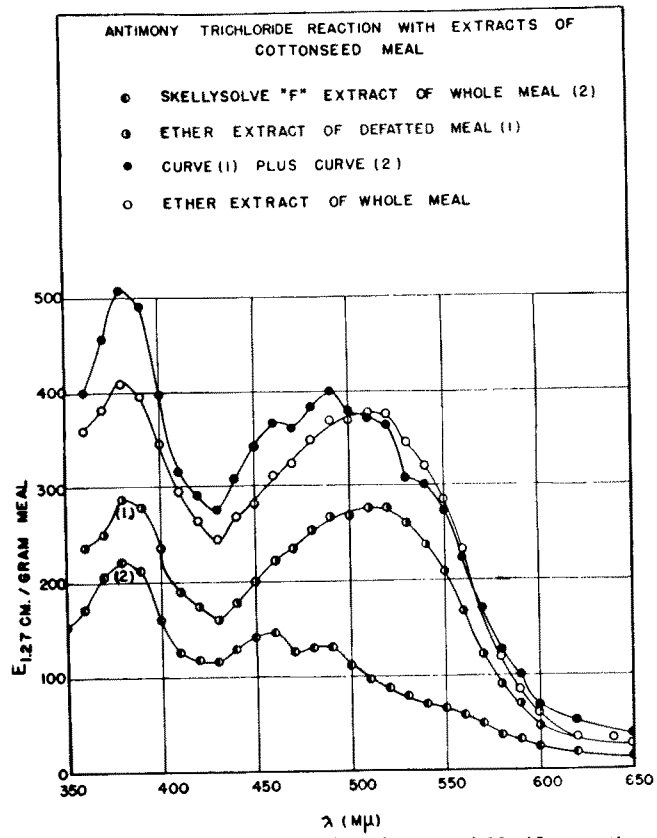

FIG. 6. Absorption spectra of antimony trichloride reaction products with extracts of cottonseed meal.

antimony trichloride to give a product having a characteristic absorption spectrum with a maximum at 450 m (mμ). It is this pigment which apparently is responsible for the color of alkali refined cottonseed oil.

Gossypurpurin, a purple pigment of cottonseed: During the purification of gossypol extracted with ether from cottonseed meal, purple and red zones were observed on the calcium phosphate adsorption columns. Elution with ethyl alcohol, after exhaustive elution with toluene, gave a dark red elutriate which showed strong absorption at 360 to 370 m (mμ) and two very much lower absorption bands at 525 and 560 m (mμ). This absorption was identified with the absorption spectrum of the so-called "red gossypol" reported by Podol'skaja (19) and later prepared and studied by Campbell, Morris and Adams (13).

Further investigation showed that it was possible to separate a purple pigment from the so-called "red gossypol." This purple pigment was found to have marked absorption bands at 525 and 560 m (mμ). As the purification progressed, there was observed a progressive increase in the ratio of the height of the maximum at 560 m (mμ) to that at 360 to 370 m (mμ) as compared with that exhibited by a preparation of "red gossypol." (See Figure 8.) Red gossypol prepared according to the method of Podol'skaja showed a ratio of 0.04, whereas in one of the highly purified samples of the purple pigment the ratio of the height of the maximum at 560 m (mμ) to the height at 360 to 370 m (mμ) was 1.64.

The "red gossypol" of Podol'skaja is a mixture of yellow gossypol and a purple pigment, and the combination of colors produces a mixture which appears red to the eye when either a concentrated solution or

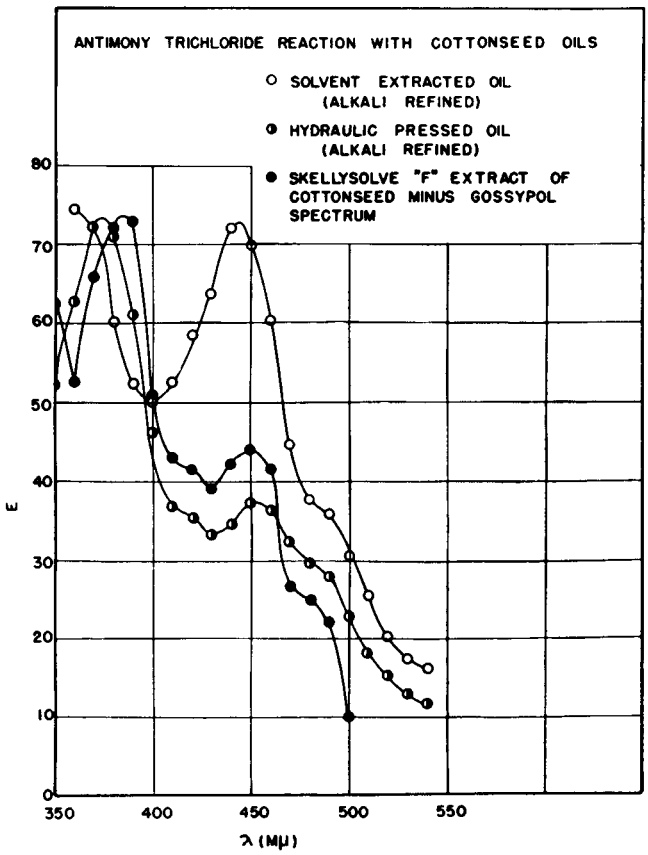

FIG. 7. Absorption spectra of antimony trichloride reaction products with cottonseed oils.

the solid is observed. Dilution of "red gossypol" produces a yellow solution as the absorption due to the slight inclusion of the purple pigment fades out and the absorption due to the gossypol preponderates. Solutions of the purple pigment are still purple in any dilutions in which any color is still observable. The change from red to yellow observed by Campbell, Morris and Adams (13) when crystals of red gossypol were crushed was found to be an optical effect corresponding to dilution and not to conversion of red to yellow gossypol as they presumed. It is probable that the preponderance of gossypol in their preparations of "red gossypol" accounts for the observations by Podol'skaja, and by Campbell, Morris and Adams that "red gossypol" undergoes all of the characteristic reactions of gossypol.

The purple pigment, for which the name "gossypurpurin" is proposed, has not yet been obtained in an absolutely pure state, but it has been possible to establish some of its physical and chemical properties. In the dry state it appears to be perfectly stable, but in solution it decomposes under various conditions to a pale yellow pigment which does not exhibit the characteristic properties of gossypol. It is rapidly decomposed by the action of light. Even in the absence of light, solutions of gossypurpurin in chloroform, ethyl acetate, diethyl ether, and dioxane may turn yellow in a few hours. The instability in these various solvents increases in the order named. In methyl alcohol, ethyl alcohol, acetone, and pyridine the yellow decomposition product is formed in a few minutes. Gossypurpurin is more susceptible to decomposition by traces of base than of acid, but it is rapidly decomposed by either if present in more than traces.

With a chloroform solution of antimony trichloride, gossypurpurin forms an unstable blue complex with a characteristic absorption spectrum having a maximum at 650 m (mμ). It gives a purple reaction with ferric chloride, reacts with bromine, and is unaffected by sodium dithionite ($Na_2S_2O_4$).

Other reactions which have been observed indicate a relationship between gossypol and gossypurpurin; but since all or any of these reactions may have been due to contamination of the product by gossypol, it is not yet possible to state the exact nature of the relationship between the two pigments. It does not appear, however, that gossypurpurin is directly convertible, *in vitro,* into gossypol.

Chromoproteins of cottonseed: Although the reaction of gossypol with protein was first postulated by Clark (6) in 1928 as a mechanism to explain the detoxification of cottonseed meal by heat treatment, the isolation of such a compound has not previously been reported. During the development of a method for obtaining gossypurpurin, a substance was isolated which has been shown to be either a mixture of gossypol-protein and gossypurpurin-protein or a complex compound of the two pigments with protein.

It has frequently been observed at certain stages of its development and storage that cottonseed contains a purple or blue pigment which can be obtained in suspension by treatment of the cottonseed meal with water. This blue pigment has recently been examined by Podol'skaja (22) whose investigations indicated it to be a pigment of the anthocyanin group.

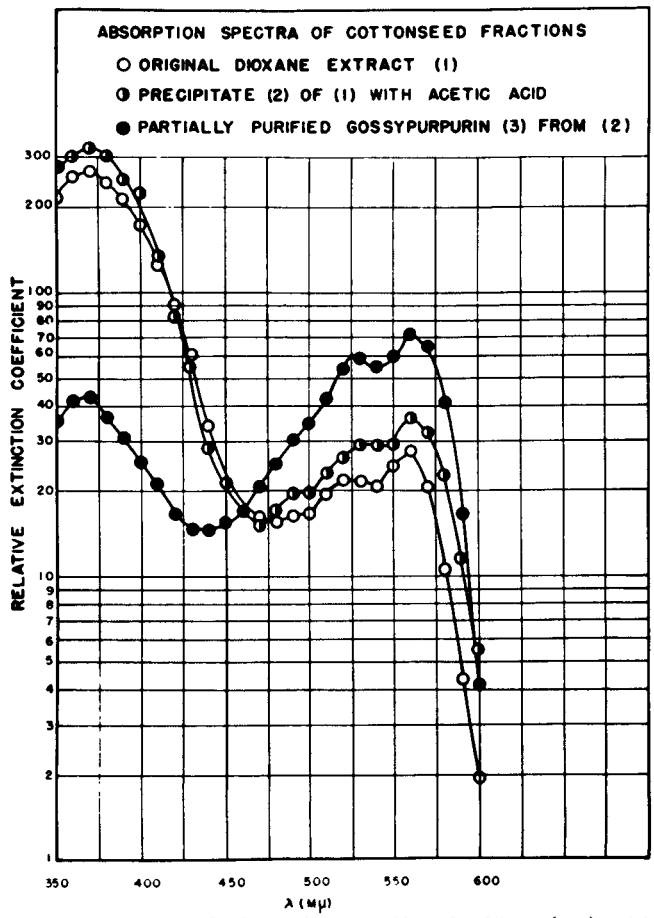

FIG. 8. Increased absorption at 560 and 525 m (mμ) with purification of gossypurpurin.

Investigation of the blue suspensoid, carried out in this laboratory, has shown it to be a compound or compounds of gossypol and gossypurpurin with protein. The protein complex, after removal of free pigment by treatment of the solid with glacial acetic acid contains 7 percent of nitrogen and is insoluble in water and chloroform.

Treatment with dioxane of the washed solid splits it into a deep reddish purple part soluble in dioxane and in chloroform and a colorless part which is insoluble in dioxane and chloroform. When the chloroform solution of the red pigment is treated with antimony trichloride the color reactions characteristic of gossypol as well as of gossypurpurin are obtained. The insoluble residue contains 12 percent of nitrogen; gives a positive biuret action; and shows a twenty-fold increase in free amino nitrogen upon acid hydrolysis. These reactions indicate that it is a protein. It is evident, therefore, that the gossypol and gossypurpurin were first obtained in combination with protein and that this complex was dissociated into the pigments and uncombined protein by treatment with dioxane.

Further evidence for the existence of the gossypol-protein complex was obtained by ether extraction of the meal which had previously been exhaustively extracted with water. That the ether extractable gossypol content had been markedly reduced is shown by the lower extinction coefficient at 510 to 520 m ($m\mu$) of the antimony trichloride-reaction product. The absorption spectrum curve is shown in Figure 4 with similar curves for the same meal which had not been previously extracted with water. Since free gossypol is not soluble in water, it must have been removed from the meal in the form of its protein complex.

It is evident that gossypol exists normally in the cottonseed in both free and combined state. Certain solvents are able to break this combination, some more efficiently than others. On the basis of recorded results, aniline is the most efficient extractant but even ether is capable of freeing gossypol from the gossypol-protein compound. These facts no doubt account for the observation by Halverson and Smith (23) that there is no sharp boundary between free and so-called bound gossypol, but that, on the contrary, as the ether extraction is continued gossypol is extracted at a progressively slower rate.

The gossypurpurin prepared by purification of the pigment mixture obtained by dissociation of the protein complex shows all of the reactions characteristic of the gossypurpurin prepared by purification of the material obtained by direct extraction of cottonseed meal with ether, dioxane or chloroform.

The gossypurpurin-protein complex is insoluble in water, and colloidal suspensions of it can be precipitated by the addition of salt. The spectroscopic examination of the water-soluble violet pigment reported by Podol'skaja which led her to the conclusion that it belonged to the anthocyanin group was probably made on a colloidal solution. Such a solution was prepared in this laboratory and was observed to have an absorption spectrum with a single maximum at 570 m ($m\mu$) instead of the maxima at 525 and 560 m ($m\mu$) observed for true solutions of gossypurpurin.

Summary

A method for the purification of gossypol has been developed which yields material differing in optical properties from those previously reported for gossypol preparations. A colorimetric test for extractable gossypol has been described.

It has been shown that cottonseed contains at least three pigments in addition to gossypol. Some of the properties of the three pigments have been reported. One of these newly detected pigments, gossypurpurin, has been shown to be the substance which, mixed with gossypol, constitutes the so-called "red gossypol" of Podol'skaja.

The frequently reported water-dispersable blue pigment of cottonseed has been shown to be either a complex of gossypol, gossypurpurin and protein or a mixture of two protein-pigment complexes. Its dissociation into the two pigments and protein has been accomplished.

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